UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

AUG 9 - 2015

MEMORANDUM

SUBJECT: University of Kentucky *Wolbachia pipientis* wAlbB *Ae. aegypti* EUP 8887-EUP-E review

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Thru: John L. Kough, Ph.D., Senior Scientist
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511P)

CONTAINS CONFIDENTIAL BUSINESS INFORMATION

ACTION REQUESTED: To review the University of Kentucky *Wolbachia pipientis* wAlbB EUP product identity, manufacturing process, product chemistry and composition, analysis and certified limits, physical and chemical characteristics, and colony feeding blood for filarial nematodes and arboviruses data to determine if the registrant has submitted adequate data to support a FIFRA section 5 experimental use permit.

CONCLUSION: Acceptable, provided that the registrant provides EPA with the following data;

1. The applicant should continue to provide reports from samples of the mosquito population used to produce male wAlbB strain *Ae. aegypti* for possible infection with pathogenic viruses such as: dengue, Eastern equine encephalitis, West Nile, other arboviruses, and lymphatic, subcutaneous, and serous cavity filariasis.

2. Address the research results reported in Dodson et al (2014), Glaser & Meola (2010), Hughes et al (2014) and Hussain et al (2013). Specifically, Dodson et al (2014) reported wAlbB infected *Culex tarsalis* may increase West Nile Virus infection rates and reduce immune gene expression of the REL1 (antiviral Toll pathway). While it is clear more research is necessary, please address this finding and how it relates to *Ae. aegypti* wAlbB releases and the potential or

probability of increased infection rates and reduced immune gene expression of West Nile Virus in wAlbB infected mosquitoes . Also, please discuss your opinion on if these findings are due to host type, *Wolbachia* strain type, and or both. Include a discussion on how other environmental factors such as temperature as described by Hughes et al (2014) may influence West Nile Virus infection rates, immune gene expression of antiviral pathways, and transmission potential. Reconcile the differences in the literature between Dodson et al (2014), Hughes et al (2014) and Glaser & Meola (2010), Hussain et al (2013) which reported increased host resistance to West Nile Virus with *Wolbachia* presence in *Ae. aegypti* and *Culex quinquefasciatus*. The differences are most likely due to difference in strain and host uses, unfortunately no report was found by EPA that specifically discusses *Ae. aegypti* wAlbB and West Nile Virus. EPA requests that you perform tests of *Ae. aegytpi* wAlbB males and females that are captured after releases to determine if *Ae. aegypti* wAlbB West Nile Virus positives are found, and determine if the virus has been enhanced relative to West Nile Virus positive *Ae. aegypti* without *Wolbachia*.

3. The applicant should monitor the release of wAlbB infected *Ae. aegypti* females (strain) at the release sites. If PCR is used to monitor for the wAlbB strain, describe how these wAlbB infected females will be distinguished from the wild *A. aegypti* females that have mated with the wAlbB infected *A. aegytpi* males. In other words, how do you make sure that the wild type females do not appear wAlbB positive (using PCR) after fertilization with a wAlbB infected male.

4. The applicant should perform sampling of released mosquitoes to confirm the rate of female mosquitoes released.

5. Trapping of *Ae. aegypti* and additional mosquitoes that co-occur at the site and are collected in traps; monitoring population density for an impact of male releases.

6. Assessment of release male quality by holding a subset in a cage and monitoring survival/fitness.

7. Mark-Release-Recapture; marking a subset of release males with dust and then monitoring for their recapture; this addresses questions of the release to indigenous male ratio and release male survival.

8. Egg collection from the field to examine for a reduction in egg hatch.

9. Monitoring of environmental conditions (temperature, wind speed, etc.) from a local NOAA station.

10. The University of Kentucky should provide statistical analysis of their data for the inferential power of their claim that 1 female wAlbB strain is expected per 250,000 individuals released. According to Calvitti et al. (2015) the current sexing technology is such that 1% female contamination is expected during male releases. Discuss how and why your sexing technology is superior to this.

11. Address the report by Calvitti et al. (2015) that fertile crosses between wAlbA low density males and ARwP females demonstrate that mosquitoes with differing *Wolbachia* strains may still be fertile, and how this finding impacts *Wolbachia* male release strategies. Discuss how the finding in this study that the risk of bidirectional CI failure should be evaluated by sampling wild type males prior to field releases. And, if pertinent, present data on sampling of male pre-releases.

~~CONTAINS FIFRA CONFIDENTIAL BUSINESS INFORMATION~~

DATA REVIEW RECORD

Active Ingredient:	*Wolbachia pipientis* wAlbB strain
Product Name:	*Wolbachia pipientis* wAlbB strain
Company Name:	University of Kentucky, Entomology (88877-EUP-E)
DP Barcode:	426685
Decision No.:	500458
MRID No:	49579401 to 49579405

BACKGROUND:

W. pipientis is an obligate intracellular bacterium that is commonly found in 65% of insects, including mosquitoes in some geographic regions of the world, and does not survive outside of hosts. *W. pipientis* has been introduced in *Ae. aegypti* through microinjection and interspecific breeding and is considered a microbial pest control agent. The presence of *Wolbachia* in a mating can cause cytoplasmic incompatibility and karyogamy failure in the zygote. No offspring are produced resulting in a limitation on reproduction when males are introduced into a population of mosquitoes that do not have *Wolbachia* present or carry different strains of *Wolbachia*. Cytoplasmic incompatibility arises because of asynchrony between the maternal and paternal pronucleus during mitosis. *Wolbachia*, has not shown any indication of being dangerous for humans. According to immunological test results, people were exposed to mosquitoes carrying *W. pipientis* did not produce antibodies against *W. pipientis*. There is no evidence *W. pipientis* has been ever transferred to a person. Recently, in French Polynesia *Ae. polynesiensis* mosquitoes infected with *W. pipientis* were released, and caused a significant reduction in egg hatch.

Mosquitoes will be shipped from the University of Kentucky to the Consolidated Mosquito Abatement district in Selma, CA. Fresno County reported 43 human cases of West Nile Virus in 2014 and *Ae. aegypti* has only recently been detected in the county. Approximately 100,000 male *Ae. aegypti* infected with *W. pipientis* WB1 (stable inherited wAlbB) strain are planned for release per week for 6 months between 2015 and 2016. The mosquitoes will be released at 4 sites in Fresno County, CA where initial population densities are estimated at 1,500 per site. The treatment area is expected to extend to a radius of 100m at each release site, which are designated as neighborhoods. Mosquito population are expected to decline and will be compared against a chosen non treated site. Sampling of mosquitoes at release sites will be performed

weekly at each identified plot within a site, using BG traps and ovicups. Releases may begin as early as the fall of 2015.

While research has shown that *Wolbachia* presence decreases a mosquito's capability to transmit dengue, recent research has shown that an artificially introduced *Wolbachia* infection in a mosquito may cause a transmitted pathogen within a mosquito to possibly increase infections rates and reduce antiviral pathways with West Nile Virus presence (Dodson et al 2014). Contrary reports by Glaser & Meola 2010 and Hussain et al 2013 have shown that increased host resistance to West Nile Virus with *Wolbachia* presence of wMelPop (but not wMel) strains in *Ae. aegypti* and *Culex quinquefasciatus* occurs. The research is not yet conclusive and more data will be needed to understand how pathogen enhancement or suppression works.

Product Identity: (MRID 49579401)

W. pipientis is found in rod-like and coccoid forms (0.5 to 1.3 um) and (0.25 to 0.5 um). It was first discovered in *C. pipiens* and has been found in 65% of all insect species, and other invertebrates, but does not infect vertebrates. *W. pipientis* is extracted from a wild type *Ae. albopictus* donor and microinjected into an uninfected *Ae. aegypti* embryo. Infected females were chosen and mated with an aposymbiotic *Ae. aegypti* male, thus creating a persistent line of *W. pipientis* infected males and females due to the maternal inheritance of *W. pipientis*. No egg hatches have been observed in the laboratory when uninfected wild type *Ae. aegypti* type females are mated with *W. pipientis Ae. aegypti* males in laboratory conditions.

Manufacturing Process: (MRID 49579402)

W. pipientis originating from *Ae. albopictus* is microinjected into the embryonic cytoplasm of *Ae. aegypti* embryos. The microinjected *Ae. aegypti* lines are designated as WB1. WB1 lines were tested using PCR to confirm the presence of *W. pipientis*. The WB1 lines are reared using standard laboratory techniques. WB1 eggs are hatched in deoxygenated H_2O, and further reared using municipal water in plastic pans. The larvae are fed powdered liver, dog, cat, and fish food *ad libitum*. Before eclusion pupae are transferred to adult cages and are initially fed a 10% sucrose solution then blood meal. After blood meal feeding, adult females will lay eggs that are collected for 3 consecutive days. After a 1 week maturation period, the eggs are submerged in deoxygenated H_2O and the rearing process is repeated. Mosquitoes in each stage of development are kept at 28+- 0.6 °C, 73$\pm$2 % relative humidity, and at a 16:8 light/dark cycle. Arthropod Containment Level - 1 procedures are followed, which does not allow for the entry or exit of mosquitoes. Several times per week WB1 strain mosquitoes are morphologically examined for confirmation of species. Every 25 generations PCR is done on 25 males and females to check for the presence of *W. pipientis* in WB1 lines. WB1 mosquitoes pupae intended for release are mechanically separated in order to remove females. Due to mechanical separation errors 1 female is released per 250,000 males. The separation device consists of an aluminum plate that supports two glass panes are separated by size. A visual sort is done to remove any residual females. The mechanical and visual sorting is based on size. Female pupae are much larger than male.

Prior to release at field sites male mosquitoes are chilled at 15 °C inside petri dishes in a cooler. When released males are placed on a platform and exposed to ambient air conditions to acclimatize, after which they disperse into the environment.

Evaluation of *Wolbachia* wAlb-B infected *Aedes aegypti* and colony feeding blood for filarial nematodes and arboviruses (MRID 49648801)

Assays of wAlbB infected *Ae. aegypti* are done using Vector Test Systems, Inc. test kits, see Table 1 below. Test are also performed monthly during planned releases to monitor for pathogenic viruses, which will be submitted in Annual EUP reports as the registrant has done previously in 2013 and 2014.

Table 1. Results of assays for pathogens in wAlbB-infected Ae. *aegypti*

			Number positive			
Date	Lot Number	Number of Mosquitoes Tested	West Nile Virus[a]	St. Louis Encephalitis[a]	Eastern Equine Encephalitis[a]	Dengue Virus[b]
6/5/2015	15-05	40	0	0	0	0

[a] – Test kit number: WSE-K050; West Nile virus, Saint Louis Encephalitis virus and Eastern Equine Encephalitis virus

[b] – Test kit number: DEN-K050; Dengue 1, 2, 3, and 4 viruses

Duplicated from MRID 49648801

DISCUSSION:

Cytoplasmic incompatibility occurs at very high frequencies in matings between *Wolbachia* harboring male mosquitoes and wild type females that do not harbor *Wolbachia*. Also, when male and female mosquitoes that harbor different strains of *Wolbachia* mate, cytoplasmic incompatibility occurs at very high frequencies. Newly published research, has indicated that in crosses of female mosquitoes with the ARwP strain and males with the wAlbB strain is 20% fertile (Calvitti et al 2015). In mosquito populations where *Wolbachia* is not naturally present, for example where dengue fever is a public health problem, releases of *Wolbachia*-infected male and female mosquitoes have been executed because the presence of *Wolbachia* reduces the mosquito's ability to transmit dengue. This strategy focuses on establishing *Wolbachia* in the mosquito population to limit dengue transmission. Recently, however, it has been reported that the introduction of *Wolbachia* wAlbB strain into *Cx. tarsalis* somatically (not through stable maternal inheritance) may increase West Nile Virus infection rates (Dodson et al 2014). Additionally, it has been discovered that somatically introduced *Wolbachia* may also enhance *Plasmodium* infections in mosquitoes (Hughes et al 2014). These research results have been based on somatic infections, and it is not known how *Wolbachia* presence in mosquitoes may suppress or enhance pathogens under stable artificially introduced maternal inheritance and in natural matings in the environment. Furthermore, factors such as temperature may dictate whether a pathogen will be suppressed or enhanced. Information on the suppression or enhancement of West Nile Virus in *Ae. aegypti* has not been reported with the exception of reports by Glaser & Meola (2010) and Hussain et al. (2013) which show that increased mosquito host resistance to West Nile Virus with wMelPop (but not wMel) strains in *Ae. aegypti* and *Culex quinquefasciatus* occurs. Therefore, it is impossible to know if enhancement is due to the

mosquito type, *Wolbachia* type strain, both, laboratory conditions, or a more complex combination of factors. Overall, it must be recognized *Wolbachia* may have the potential to inhibit one parasite and enhance another, therefore caution should be exercised when implementing a *Wolbachia* biological control strategy (Dodson et al 2014). This cautionary approach is especially important when more than one parasite species occurs or may occur (Hughes et al, 2014).

The current EUP is for Fresno County, CA. From 1999 to 2014 California has reported 4,816 cases and Fresno County averages .5 to .99 cases per 100,000 persons which is average for the state for West Nile Virus cases per year (http://diseasemaps.usgs.gov/mapviewer/). However, in 2014 Fresno County reported 43 cases of West Nile Virus, which was the third highest number of cases in California.

The registrant has claimed that 1 female *Ae. aegypti* with wAlbB *Wolbachia* is released per 250,000 males with *Wolbachia*. With such a female release rate, a stable *Ae. aegypti* population with *Wolbachia* could be established that may enhance West Nile Virus presence in mosquito populations. However, according to Calvitti et al (2015) the sexing technology allows for at least 1% female contamination.

Overall, more research is necessary to understand the risk involved with *Wolbachia* infected *Ae. aegypti* releases for both establishment of a new *Wolbachia* strain in the mosquito population and potential enhanced virus transmission. In order to fully understand the risk involved with wAlbB *Ae. aegypti* releases, mating experiments must be conducted with stable maternal wAlbB *Ae. aegypti* carrying West Nile Virus to understand if the virus is enhanced or suppressed and what environmental factors may impact enhancement or suppression.

CONCLUSION: Acceptable, provided that the registrant provides EPA with the following data;

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References

Calvitti M, Marini F, Desiderio A, Puggioli A, Moretti R. 2015. Wolbachia density and cytoplasmic incompatibility in Aedes albopictus: Concerns with using artificial Wolbachia infection as a vector suppression tool. PloS one 10: e0121813
CDC.gov, http://diseasemaps.usgs.gov/mapviewer/

Dodson BL, Hughes GL, Paul O, Matacchiero AC, Kramer LD, Rasgon JL. 2014. Wolbachia enhances West Nile Virus (WNV) infection in the mosquito *Culex tarsalis*. PloS one 8: e2965

Glaser RL, Meola MA. 2010. The native Wolbachia endosymbionts of Drosophila melanogaster and Culex quinquefasciatus increase host resistance to West Nile virus infection. PloS one 5: e11977

Hughes GL, Rivero A, Rasgon JL. 2014. Wolbachia can enhance Plasmodium infection in mosquitoes: Implications for malaria control? PloS one 9: e1004182

Hussain M, Lu G, Torres S, Edmonds JH, Kay BH, et al. 2013. Effect of Wolbachia on Replication of West Nile virus in a mosquito cell line and adult mosquitoes. Journal of virology 87: 851-8